Original Article

Evaluation of the efficacy of AgNOR as a proliferative marker in oral leukoplakia: A morphometric analysis

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ABSTRACT

Background: Silver stainable nucleolar organizer regions (AgNORs) are replicatory markers which may have a place in objectively characterizing dysplasia. **Materials and Methods:** A study of various morphometric parameters related to AgNORs was performed in basal and parabasal layers of normal human oral epithelium, nondysplastic leukoplakia, and dysplastic leukoplakia employing photomicrographs of silver stained paraffin embedded sections using image analysis, to assess the usefulness of these parameters in distinguishing dysplastic leukoplakia from nondysplastic oral leukoplakia. **Results:** Out of various mean AgNOR related parameters, AgNOR count, area, perimeter, and proportion were found to be higher in dysplastic leukoplakia as compared to nondysplastic leukoplakia. On statistical analysis, AgNOR count showed statistically significant differentiation between dysplastic and nondysplastic leukoplakia. While other parameters can distinguish normal oral epithelium from dysplastic and nondysplastic leukoplakia. **Conclusion:** To conclude, the AgNOR count is the most appropriate marker to differentiate between dysplastic and nondysplastic leukoplakia.

Key words: Argyrophilic nucleolar organizer regions AgNOR, leukoplakia, morphometry

Introduction

In the long intervening period between initiation of carcinogenic tobacco habits and the development of invasive oral cancers, well-defined oral potentially malignant lesions may occur, of which leukoplakia is the most common. Leukoplakia is a white plaque of questionable premalignant risk, having excluded other known lesions that carry no increased risk for cancer,[1,2] with histological presentation ranging from mild hyperkeratosis to squamous cell carcinoma.[3]

More than 75% or oral cancers are reported to occur in a preexisting leukoplakia and the prevalence of leukoplakia in the Indian population is reported to range from 0.7 to5.0% in various regions in India[4] and the global prevalence of leukoplakia is 2.6%.[5]

Because of their varied histological presentations, it is important to distinguish between these lesions and assess the associated risk in order to determine the clinical management and predict prognosis. Currently the severity of these lesions is usually assessed by histological demonstration of epithelial dysplasia in biopsy samples. To date, no clear markers for grading of epithelial dysplasia have evolved, and histological criteria for diagnosing a 'dysplastic' lesion are still subjective. In absence of any objective criteria, there is wide variation of opinions between oral pathologists in diagnosing such lesion.[6]

It is known that hypertrophy of the nucleolus is one of the most distinctive cytological features of cancer cells. Dysplastic cells more frequently display a larger nucleolus

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than benign cells and nucleolar size might represent morphological parameters of the cell proliferation rate in cancer tissue.[6]

Nucleolar organizer regions (NORs) are loops of deoxyribonucleic acid (DNA) that transcribe to rRNA,[7] and the nucleolus is a structure containing this chromosomal part and in addition the material which accumulate around the NOR, most notably the ribosomal ribonucleic acids (rRNAs) and their precursors as well as specific ribosomal proteins.[8] The qualitative or quantitative changes in interphase NORs may be visible in relation to proliferative activity or transformation, and hence could aid diagnosis or prognostication of malignancy.[9] NORs and associated proteins show affinity for metallic silver and can be visualized by a one-stage argyrophil (AgNOR) method, by staining NOR associated proteins.[10]

High AgNOR counts have been found to reflect proliferative status of cell[11] and correlate with poor prognosis in malignant conditions.[12]

AgNOR quantification by image cytometry has proven useful which indicates marked changes from the basal to the upper malpighian layers in tissue with functional polarity (both normal and pathological) would support an association between differentiation linked cellular activities and NOR patterns.[13] Most of these studies employed image analysis to express AgNORs in number and size.[11]

The present study used morphometry to evaluate NOR related parameters including AgNOR count, AgNOR area, AgNOR perimeter, and AgNOR proportion (Mean AgNOR area/mean nuclear area) in normal oral epithelium, dysplastic and nondysplastic leukoplakia and significance of these morphometric parameters in distinguishing nondysplastic leukoplakias (NDLKs) from dysplastic ones.

Materials and Methods

The study sample included biopsy specimens from 50 cases of oral leukoplakia (22 nondysplastic, 28 dysplastic) and 10 specimens of normal oral epithelium. The control tissues were taken from third molar impaction. Ethical clearance was taken from ethical committee of the institution. All specimens were collected from the archieves of the Department of Oral Pathology and Microbiology, Saraswati Dental College and Hospital, Lucknow. The diagnoses were reviewed using routine hematoxylin and eosin stained sections and were considered as 'gold standard' for comparing with the AgNOR based diagnoses. The histopathological grading was made using world health organization (WHO) criteria for potentially malignant lesions. Based on the diagnosis the cases were divided into two groups. The first group, named as nondysplastic dysplasia (NDLK) consisted of lesions diagnosed as hyperkeratosis, epithelial hyperplasia and mild epithelial dysplasia. The second group, named as dysplastic leukoplakia (DLK) consisted of moderate epithelial dysplasia, severe dysplasia and carcinoma *in situ*.

Following this, the tissue sections were stained for AgNOR using ploton technique: For each case, 3 μm thick sections of routinely processed specimens from formalin fixed, paraffin-embedded blocks were dewaxed in xylene and dehydrated through alcohols to deionized water. The sections were then incubated in dark for 30 min at room temperature (25°C) in a fresh solution made by mixing two parts of 2% gelatin in 1% formic acid with one part of 50% aqueous silver nitrate solution. The sections were then washed in running deionized water, dehydrated in ascending alcohol concentrations, cleared in xylene, and mounted with DPX. The AgNORs were visualized as intranuclear brown to black dots of different sizes under light microscopy.

Microscopic fields, representative of the lesion, were identified and photographs were taken using Olympus Live View Digital SLR Camera E-330 in 46 different fields moving from left to right to avoid any overlapping of cells. The best four photographs regarding the picture quality were selected for each case. The photographs were analyzed using Image Pro Express 6.0 for windows, (Media Cybernetics) after calibrating the software with photomicrograph of stage micrometer. AgNORs from 100 randomly selected nuclei of epithelial cells in basal and parabasal layers were assessed in four different fields at 100X magnification for their numbers, area, and perimeter using magic wand and trace wand tool of image analysis. Similarly nuclear area was also analyzed. The obtained results were used to calculate the various indices as explained below.

Calculation of morphometric parameters

Mean AgNOR Count/Nuclei = Total AgNOR count/100
Mean AgNOR Area (μm^2)/Nuclei = Total AgNOR area/100
AgNOR Proportion = Mean AgNOR area/mean nuclear area.

The results were statistically analyzed for relationship between AgNOR count and other AgNOR related parameters in normal oral epithelium, NDLK and DLK.

Statistical analysis

One way analysis of variance (ANOVA) was performed as to compare the mean AgNOR parameters among normal epithelium, NDLK, and DLK to determine

the differences among the three groups. For statistical evaluation, a prior significance level was set at 0.05 levels. Owing to unequal sample size, pairwise comparison test was performed. The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical analysis software.

Results

The results of the present study [Table 1] show mean AgNOR/nucleus of different groups in various AgNOR parameters like count, area, perimeter, and proportion reveals increase in these parameters of AgNOR in DLK indicating an order of normal < non-dysplastic < dysplastic. Hence, minimum value is seen in control group and maximum in DLK.

Tables 2 demonstrates the results of the ANOVA "F" test which were used to compare the values in between and within groups, as well as it also shows the *P* value. From this Analysis of variance revealed statistically significant difference (p) for AgNOR count (< 0.001), AgNOR area (0.004), AgNOR perimeter (0.015) and AgNOR proportion (0.027) indicating increase in values from control to DLK. Hence, reveal increases in proliferation rate from control to DLK.

Table 3 shows intergroup differences for AgNOR parameter in control and leukoplakia groups using pairwise student "*t*" test and "*p*" test. The results revealed that there was a statistically significant difference in mean AgNOR count AgNOR area, AgNOR perimeter while the proportions were not statistically significant. Further, comparison of these similar parameters was done between NDLK and DLK. The results showed that only AgNOR count showed statistical difference between these two groups.

Discussion

The presence of dysplastic areas in the epithelium is believed to be associated with a likely progression to cancer. When architectural disturbance is accompanied by cytological atypia (variation in size and shape of keratinocytes) the term dysplasia applies.[14] It should be emphasized that dysplasia is a diagnosis defined by the presence of certain histological and cytological features. Ideally, the diagnosis corresponds to the nature of the lesion meaning that a mucosa with epithelial dysplasia has an increased risk of developing into carcinoma when compared to normal mucosa.[14]

Leukoplakia as a clinical diagnosis may have varied histological presentations ranging from mildly hyperkeratotic lesions to those exhibiting severe dysplasia.[14] Many oral leukoplakias regress or stay quiescent, but many progress and about 3-6% turn into squamous cell carcinoma in future.[15] It is, therefore, important to distinguish between these lesions as their management may differ. Mildly, hyperkeratotic/hyperplastic lesions may be kept under observation and may resolve spontaneously, whereas dysplastic lesions need to be excised.[14]

Image analysis provides a unique advantage of reducing some of the difficulties by giving the operator

Table 1: Calculation of mean AgNOR count/nucleus in various parameters in all the groups including control and leukoplakia (NDLK and DLK)

Mean ± SD	AgNOR count	AgNOR area	AgNOR perimeter	AgNOR area/nuclear area
Control	0.927 ± 0.870	2.699 ± 1.513	8.35 ± 3.52	0.058 ± 0.026
NDLK	1.28 ± 0.133	3.579 ± 0.707	10.74 ± 1.90	0.080 ± 0.020
DLK	1.605 ± 0.321	4.257 ± 1.469	11.89 ± 3.81	0.088 ± 0.036

NDLK: Distinguishing nondysplastic leukoplakias, DLK: Dysplastic leukoplakia, AgNOR: Argyrophilic nucleolar organizer regions

Table 2: Calculation of analysis of variance of AgNOR in various parameters in between the groups

	AgNOR count		AgNOR area		AgNOR perimeter		AgNOR area/nuclear area	
	"F"	*"P"*	*"F"*	*"P"*	*"F"*	*"P"*	*"F"*	*"P"*
ANOVA '*F*'	32.600	0.001	6.020	0.004	4.553	0.015	3.848	0.027

AgNOR: Argyrophilic nucleolar organizer regions

Table 3: Intergroup differences for various AgNOR parameters in between two groups i.e., control versus leukoplakia and within leukoplakia group i.e., NDLK versus DLK

Intergroup difference	AgNOR count		AgNOR area		AgNOR perimeter		AgNOR area/nuclear area	
	"t"	*"P"*	*"t"*	*"P"*	*"t"*	*"P"*	*"t"*	*"P"*
Control vs leukoplakia (NDLK + DLK)	5.538	<0.001	2.841	0.006	2.727	0.008	2.612	0.925
NDLK vs DLK	4.42	<0.001	1.987	0.053	1.288	0.204	0.011	0.360

T=Student *t*-test; *P*<0.05 significant; NDLK: Distinguishing nondysplastic leukoplakias, DLK: Dysplastic leukoplakia, AgNOR: Argyrophilic nucleolar organizer regions

opportunity to control the magnification and resolution of the images: hence reducing the errors in counting of nonspecific silver stains. It also provides a permanent record of the stained tissue and helps in assessment of various morphological characteristics of NORs which is not possible by manual counting. The literature search revealed a very few studies on oral dysplasia using morphometry which made the present study a pioneer attempt in this field.

As seen from the results of this study, the mean AgNOR count per nucleus was found to be higher in patients with DLK [Figure 1] as compared to NDLK [Figure 2] and controls [Table 1]. The AgNOR count showed statistically significant difference on comparison between group and within group [Table 2, $P < 0.001$]. These findings are in concordance with previous studies done by Warnakulasuriya and Johnson,[16] Gomez *et al.*,[17] and Chattopadhyay *et al.*,[18] who stated that mean AgNOR count increased gradually from normal epithelium to nondysplastic to DLK to squamous cell carcinoma. These higher counts found in many carcinomas were due to dispersion of AgNORs within the nucleoplasm. Higher and more dispersed counts represent dysplastic lesions at greater risk of malignant transformation.[16] The results of this study support the view that in dysplastic tissue, chromosomal disarray with multiple nucleoli appears to result in an increase in AgNORs, and higher AgNOR counts suggesting a poor prognosis in oral cancer.[19] The increase in AgNOR number and area could be the expression of an alteration of the mechanism controlling cellular proliferation and perhaps cellular differentiation. This is in accordance with the high values of AgNOR in severe and even moderate dysplasia and could represent a marker of proliferative cell hyperactivity, therefore may be a possible indication for a strict clinical management and/or a more incisive treatment of preneoplastic lesions. Neoplastic cells generally exhibit a rise in the synthesis of normal and abnormal products; hence there is a significant rise in AgNOR material. AgNOR counts rise with increased cell ploidy, with increased transcriptional activity and in stages of active cell proliferation.[20]

Apart from the AgNOR counts, various other morphometric parameters of AgNOR were also analyzed in this study. These included mean AgNOR area/nucleus, mean AgNOR perimeter/nucleus, and AgNOR proportion.

From the results of this study, it was observed that the mean AgNOR area/nucleus [Figure 3] was higher in patients with DLK as compared to NDLK and controls [Table 1], [Figure 4]. When intergroup comparison [Table 3] was made, it is seen that mean AgNOR area/nucleus was able to differentiate control group from leukoplakia group ($P = 0.006$) but was not able to differentiate NDLK from DLK ($P = 0.053$). Increased AgNOR areas with increasing grades of malignancy have been reported in non-Hodgkins lymphoma,[11] in colonic tumors,[21] and melanocytic neoplasm of skin.[22]

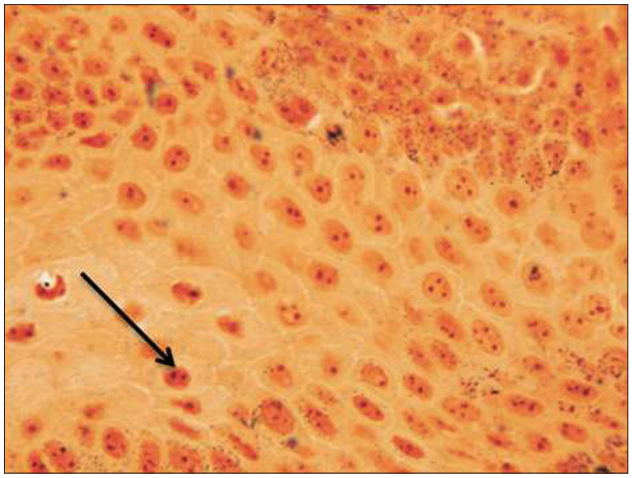

Figure 1: AgNOR staining in dysplastic leukoplakia (arrow showing AgNOR dots)

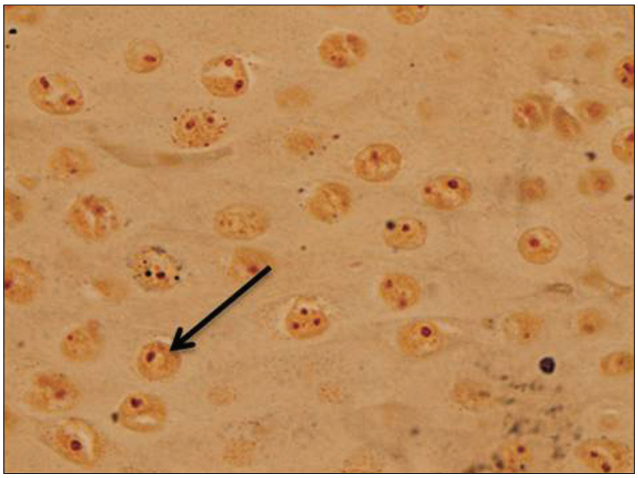

Figure 2: AgNOR staining in nondysplastic leukoplakia (arrow showing AgNOR dots)

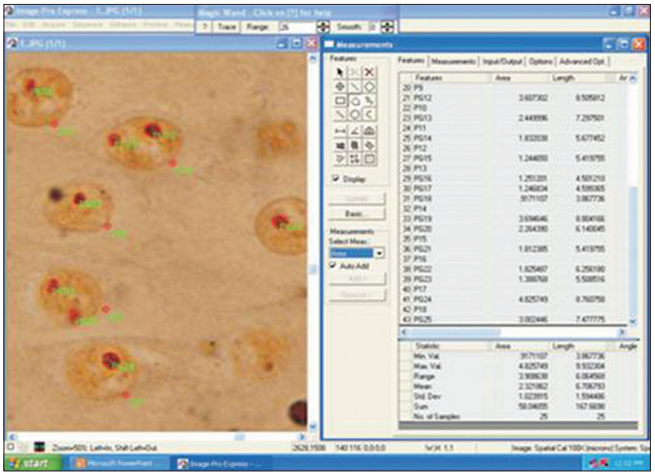

Figure 3: Photomicrograph showing analysis of AgNOR area through image pro express

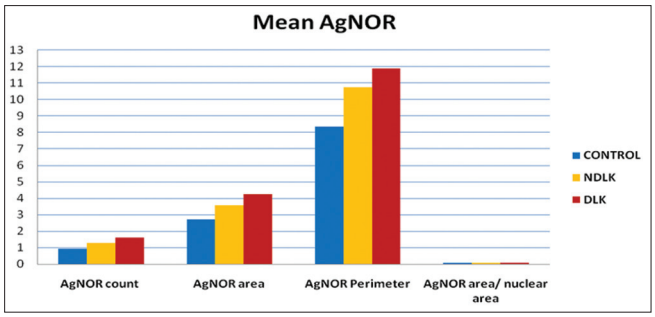

Figure 4: Mean AgNOR count in various parameters

Similar findings have also been reported in oral cavity by Muzio *et al.*,[23] who found increased mean AgNOR area/nucleus in patient with moderate and severe dysplasia as compared to mild dysplasia. Cabrini *et al.*,[24] also reported increasing AgNOR area from normal oral epithelial to benign lesions and to SCC. Derenzini *et al.*, evaluated nucleolar size, using silver staining, in 10 tumor cell lines and found that nucleolar size can reliably indicate the rapidity of cell proliferation. They postulated that the higher AgNOR protein value corresponds to a worse clinical outcome, and since nucleolar size is a representation of AgNOR protein value; thus it could be used as a parameter to assess rate of proliferation and prognosis in individual tumors.[6] The results of our study are consistent with findings of Muzio *et al.*, who found that the high value of AgNOR area in oral dysplasia could be a risk marker which could help in identifying the subgroup of lesions with worse prognosis.[23]

Another variable analyzed in our study was mean AgNOR perimeter and was found to be higher for dysplastic cases as compare to nondysplastic cases [Table 1], [Figure 4]. When the various groups were analyzed with each other [Table 3] a statistically significant difference was found between the control and leukoplakia group ($P = 0.008$), though AgNOR perimeter failed to differentiate dysplastic from nondysplastic lesions ($P = 0.204$). Perimeter of AgNORs will also be higher in cases of numerous, small, fragmented, and scattered NORs as compared to lesser and larger NORs in a nucleus. Hence, increase in AgNOR perimeter per nucleus is indicative of fragmented and more irregular NORs. Cabrini *et al.* suggested that increase in number and irregularity of NORs and a decrease in their size would be an expression of altered proliferation and differentiation mechanism in conjunction with synthesis of new oncogenic proteins present in carcinomas.[24] Hence, smaller and more irregular NORs are expected in dysplastic epithelium as compared to normal epithelium.

When the volume fraction of AgNOR per nuclei was assessed, it gave an indication of proportion of nuclear volume occupied by AgNOR. Significantly, higher values were obtained for leukoplakia (DLK and NDLK) patients as compared to controls [Table 1], [Figure 4], though this difference was statistically significant [Table 2], ($P = 0.027$) but in intergroup comparison, no significance was seen when differentiating nondysplastic from dysplastic group [Table 3], ($P = 0.360$). These findings are in agreement to those in some of the previous studies by Weeks *et al.*,[25] and Nyska *et al.*,[21] who found that the ratio of AgNOR area/nuclear area increases from control to benign and benign to malignant tissue. Other authors hypothesized that the ratio obtained more accurately reflected the proliferative status of a cell.[11] On the other hand, Cabini *et al.*, failed to establish statistically significant difference in mean AgNOR area/nuclear area.[24]

Thus, the results of the present study show a linear relationship between the various AgNOR parameters and increasing grades of dysplasia as assessed by image analysis. The morphometric parameters show a positive trend. Their role in diagnosis of epithelial dysplasia is still subject to further investigation.

In summary, results indicate a change in the NOR pattern in oral mucosal lesions (dysplastic and nondysplastic leukoplakia) when compared with normal oral epithelium. The present study reveals that mean AgNOR parameters including count, area, perimeter, and proportion were found to be increasing in dysplastic lesions when compared with nondysplastic and control group. Only AgNOR count showed statistically significant differentiation between dysplastic and nondysplastic lesions.

To conclude, the computerized morphometry of AgNOR related parameters could be valuable tool for defining objective criteria for diagnosis/determination of dysplasia in oral leukoplakia. We suggest larger studies of this nature to determine accurate, effective, and meaningful cutpoint to distinguish between NDLK and DLK.

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